Chapter I

General Introduction

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Abstract

Chitin is a natural polymer, which is globally produced in large quantities. Its degradation and assimilation is mainly a microbial process that operates through secreted enzymes. These enzymes break down the chitin fibers to short-chain oligomers recyclable in the carbon and nitrogen cycles. The use of chitin and its derivatives as recyclable and non-toxic materials has spurred research on chitinolysis in natural settings in the last years. Investigation of those natural habitats that are known to constitute a rich source of enzymes and are easy to obtain and manipulate (e.g. soil) will lead to a better understanding of the ecology of natural chitinolysis but will also offer conditions for further biotechnological applications. The research project presented in this thesis addressed the chitinolytic process in natural microbial systems starting from (1) the perceived need of answering open questions pertaining to the ecology of chitin-degrading organisms in different habitats, and (2) the industrial demand of new enzymes for chitin purification and improved fiber production.

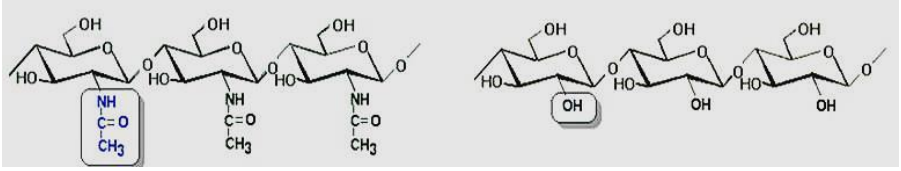
Metagenomic analysis of microbial communities with respect to their "chitinase gene pool" was employed in order to gain more details on the ecology of chitinolysis. The succession of organisms that are active on chitin (in field and laboratory conditions) was assessed to provide information on the conditions under which particular chitin degraders thrive, their potential interactions and the mechanisms behind the chitinolytic function. Exploration of microbial systems that are active on chitin thus can reveal the importance of the already known (abundant) chitin-degrading organisms, as well as the ecological and biotechnological potential of the less abundant ones.

Chitin – a polysaccharide of global importance

Chitin (the Greek word for "envelope") is one of the most predominant polysaccharides in nature. After cellulose, it is on the second place in biological turnover and it is an important component of many organisms from different taxonomic groups (Chater *et al*., 2010; Gooday, 1990a;). Thus, chitin is the main component of the cell walls of fungi, the exoskeletons of arthropods (e.g. crustaceans such as crabs, lobsters, and shrimps) and insects, the radulas of molluscs and the beaks of cephalopods and nematodes. Chitin has not been identified in prokaryotes (with the exception of *Streptomyces* spore walls), plants and vertebrates (Agullo *et al*., 2003; Keyhani *et al*., 2000; Smuker & Pfister, 1978).

Chitin was described for the first time in 1811 by the French chemist H. Braconnot, even before the description of cellulose (which was in 1838). In the light of its polymeric structure, it constitutes a fibrous material of considerable strength. However, in spite of the early discovery, chitin received relatively limited attention over the years, while extensive research and development focused on cellulose. In the 1930s, the development of nylon started a new era of application of "man-made fiber" (e.g. nylon, polyester and polypropylene), which actually hampered the further development of chitin fibers. However, recently the interest in chitin has revived, as it has clear medical and agronomical applications. Thus, the 21st century seems to constitute a "new era" for chitin, chitin derivatives and enzymes involved in chitin metabolism and degradation.

Chemically, chitin is a homopolymer of unbranched chains of ß-(1,4)–N-acetyl-D-glucose-2-amine (it is also named 2 acetylamino-ß-(1,4) glucose; N-acetylglucosamine, GlcNAc). Chitin and cellulose (ß–(1,4)-D glucosamine) have a similar structure (Figure 1).



Chitin ***Cellulose***

Figure 1. Structure of chitin and cellulose.

Chitin has a crystalline structure and it constitutes a network of organized fibers. This structure confers rigidity and resistance to attack in the organisms that contain it. X-ray diffraction studies have revealed three polymorphic chitin forms which differ in the orientation of the micro-fibrils: α-, ß-, and γ-chitin. These three forms differ mainly in the degree of hydration, the number of GlcNAc repetitive units and the orientation of chains per unit (Jang *et al*., 2004; Kramer & Koga, 1986; Rudall & Kenchington, 1973;

Vermeulen & Wessels, 1986). Pure chitin is fully acetylated and it is rarely found in nature. Rather, natural chitinous matter is only partially acetylated, leaving some parts of the matrix relatively acetyl-free (fully deacetylated chitin is called chitosan). In natural samples (primarily in marine organisms), the acetylation degree is typically 80-95% (Kumar & Ravi, 2000). The generally accepted cutoff of acetylation to distinguish between chitin and its deacetylated form chitosan (Figure 2) is 30-40%. Chitin is always present in nature in association with other molecules such as glucans, lipids or proteins.

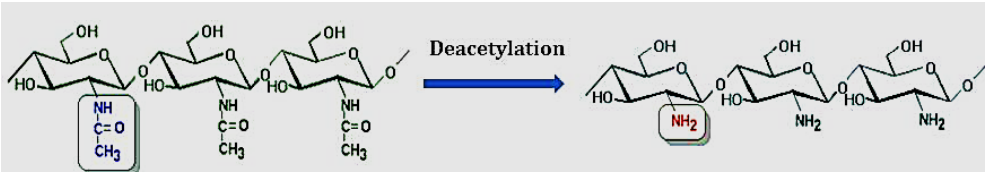


Figure 2. Structure of chitin and its deacetylated form, chitosan.

Important characteristics of chitin are its insolubility in water and its resistance to acid, alkali and many organic solvents. Chitin is the basic raw material for products such as chitosan, chitin-oligosaccharide, D-glucosamide, D-glucosamidehydrochloride and GlcNAc.

Natural production and further processing of chitin

The annual natural production of chitin is enormous, amounting to at least $\approx 10^{11}$ tons/ year if only marine chitinous waste is considered. The exoskeleton of crustaceans is currently the main industrial source of chitin. Crustaceans contain mostly α–chitin, which is by far the most common and widely available form of chitin. In contrast to shell mollusks, which constitute a less pure source of chitin, squid skeletons contain 40% chitin, which is free of calcium salts. Squid pen is the only important source of β-chitin. However, fungal chitin has been suggested to have a number of advantages compared to crustacean chitin, due to the uniform composition of the raw material and the lack of a required demineralization step during extraction (Agullo *et al.*, 2003). Usually, chitin is extracted by acid treatment (to dissolve the calcium salts) followed by alkaline extraction (to precipitate the proteins), and depigmentation (to obtain a colorless product).

Chitosan is prepared by hydrolysis of the acetamide groups of chitin. In general, two major methods for preparing chitosan from chitin with varying degree of acetylation are used. These are (1) heterogeneous deacetylation of solid chitin and (2) homogeneous deacetylation of pre-swollen chitin in an aqueous solution. In both processes, the deacetylation involves the use of alkaline solutions and long processing times.

Applications of chitin

The study of chitin and derivatives is of interest in the light of the potential of such compounds for industrial and agricultural applications. Chitin and chitosan are biodegradable, non-toxic and biocompatible materials. As we have seen, both materials can be produced from natural sources at relatively low cost (Jayakumar *et al*., 2011). Moreover, the physicochemical features of the chitin polymer allow structural modifications and thus generation of different forms. Such novel forms are in demand by biomedicine and the pharmaceutical industry. For instance, chitin fibers and congeries are used as adjuvants in wound dressings (Fan *et al*., 2009; Tamura *et al*., 2011a), scaffolds for growth in cartilage replacement (Suzuki *et al*., 2008) and support for mesenchymal stem cells in regenerative medicine (Shalumon *et al*., 2009). In addition, they may serve as nanoparticles for internal drug delivery (Huang *et al*., 2009). Chitin and chitosan can also be used in industry in the recovery of different organic molecules (Shahidi *et al*., 1999), for water purification (Zemmouri *et al*., 2012) and for removal of metals from industrial waste (Kumar &Ravi, 2000). Another important application is in agriculture, where the amendment of soils with chitin and/or chitosan has been reported as an enhancer of soil suppressiveness toward plant pathogens (Green *et al*., 2006; Radwan *et al*., 2012; Sarathchandra *et al*., 1996). Moreover, the inclusion of chitin and/or chitosan in the list of food additives has been considered in 2003 by the Codex Alimentarius Commission (Report of the 31st session of Codex Alimentarius Commission, 2009). However, these compounds are currently not listed in the General Standards for Food Additives (Codex General Standard for Food Additives, last revision 2012) nor have they been authorized as a food ingredient in the European Union.

Enzymatic degradation of chitin: chitinases and chitin deacetylases

Despite the immense annual production in natural systems, chitin does not accumulate in the environment. It is well established and accepted that degradation by released enzymes is the main mechanism by which chitin is transformed in natural systems (Hoell *et al*., 2010). The enzymatic conversion of chitin can occur through two major pathways:

(1) hydrolysis of (1-4)-β-glycosidic bonds – the chitinolytic process, and

(2) deacetylation (Figure 3).

Chitinases and chitin deacetylases are the key enzymes that attack chitin. The former group generates chitin-oligoccharide chains, which are further amenable to different metabolic processes, whereas the second group cleaves off the acetyl moiety. Both groups of enzymes have been the subject of several classification systems. The most used system is the one based on amino acid sequences provided by Carbohydrate-Active Enzymes database (CAZy; Henrissat, 1991). It is important to note that the study of the mode of action of chitinolytic enzymes directly on chitin is very difficult due to the limitations of current

biochemical techniques. Moreover, the intermediate soluble oligomers are better substrates than the insoluble polymer which can interfere with the visualization methods that are applied (Hoell *et al*., 2010).

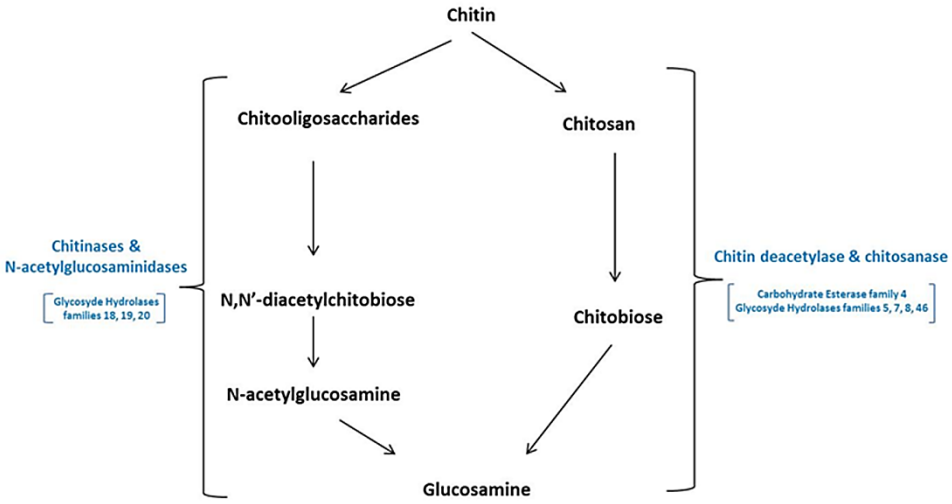


Figure 3. Enzymatic degradation of chitin – summary of enzymes and chitin derivatives.

Chitinases are glycoproteins with a wide range of molecular weights (MW) (20-115 kDa), optimal temperature of functioning (18-90oC), pH values (2-10.5), and pI values (3.5-8) (http://www.brenda-enzymes.org/). Collectively, chitinases belong to the glycoside hydrolase (GH) group, which is divided in different families. The majority of chitinases have been found in GH families 18, 19 and 20. These GH families are diverse in evolutionary terms and contain enzymes which have high substrate specificities, including enzymes with a specific chitinolytic activity (Table 1) (Henrissat & Davies, 2000). Although chitinases from families 18 and 19 have often been isolated from the same source, they do not share extensive amino acid sequence similarity. Moreover, they have completely different three-dimensional (3-D) structures and catalytic molecular mechanisms and are therefore likely to have evolved from different ancestors (Hoell *et al*., 2006; Suzuki *et al*., 1999).

Table 1. Glycoside hydrolases involved in chitin degradation (www.cazy.org/index.html).

GH family	*Enzyme*	*Nomenclature (EC)*	*Sourse (prevalence)*[x]
18	chitinase	EC 3.1.1.14	Archaea (41), Bacteria (3057), Eukarya (2103), Viruses (165)
	endo-β-N-acetylglucosaminidases	EC 3.2.1.96	
	xylanase inhibitor, concanavalin B, narbonin	non-catalytic proteins	
19	chitinase	EC 3.1.1.14	Bacteria (622), Eukarya (702), Viruses (147)
20	β-hexosaminidase	EC 3.2.1.52	Archaea (4), Bacteria (1100), Eukarya (220)
	lacto-N-biosidase	EC 3.2.1.140	
	β-1,6-N-acetylglucosaminidases – unspecific chitinolytic function	EC 3.1.1.14	

[x]CAZy database , July 2013

Ecological significance of chitin-degrading enzymes

The turnover of chitin is essential for the recycling of carbon and nitrogen in aquatic and terrestrial ecosystems. In the process, degradation by enzymes plays a critical role. Thus, chitinases and chitin deacetylases occur in a wide range of organisms, including bacteria, archaea, fungi, insects, plants and animals. The roles of these enzymes are diverse, given that different organisms produce different types of chitinases for different purposes. This is based, in the majority of cases, on the physiology and use of chitin by these organisms. In bacteria and archaea, chitinases are produced to meet nutritional needs and for parasitism on chitin-containing hosts. In arthropods, chitinases are involved in cuticle turnover and mobilization and nutrient digestion. In fungi and yeasts, chitinases are important in morphogenesis and in the degradation and mobilization of organic matter, as well as in the antagonism towards competitors. The function of chitinases produced by vertebrates is still uncertain. Recent studies have shown a role of chitinase-like proteins, particularly acidic mammalian chitinase, in inflammation, tissue remodeling and injury

(Lee *et al*., 2011). Recent advances in chitin degradation research indicate that the highest quantity of this biopolymer is turned over by microorganisms (bacteria and fungi) in both marine and terrestrial ecosystems. As a consequence, it is felt that a deep investigation of the microbial sources of chitin-degrading enzymes that occur across ecosystems will yield important new data that are of use to foster our understanding of the natural chitinolytic process.

Bacterial chitinases

Many types of bacteria obtained from natural settings present chitin hydrolytic activity. Indeed, an investigation based on cloning and sequencing of chitinase genes directly from natural habitats (e.g. marine) suggested the presence of large pools of chitin-degrading bacteria (Cottrell *et al*., 2000; Howard *et al*., 2003; Svitil *et al*., 1997). Also, studies of bacterial communities in different types of soil indicated that a diversity of chitinases was present across the investigated soils (Hjort *et al*., 2010; Metcalfe *et al*., 2002a,b). Bacteria typically possess multiple, usually inducible, chitinases, which are produced and secreted to, presumably, meet their nutrional needs. Relatively little is known about the genetic organization of chitinase genes and their regulation in the majority of chitinolytic bacteria (Delpin & Goodman, 2009; Li & Roseman, 2003). More than in other organisms, for bacteria a complex combination between different regulatory factors from the environment and gene regulation and transfer seems to play a decisive role in the selection of the best performing chitinolytic mechanism (LeCleir *et al*., 2004; Li & Roseman, 2003; Nazari *et al*., 2011).

In particular bacteria from marine environments have developed efficient systems for the depolymerization, transport and metabolism of chitin and its derivatives. This is because of their permanent contact with the so-called "rain of chitin" (provenient from degrading crustaceans) present in that environment (Howard *et al*., 2003). Also, different chitinovorous bacteria from diverse water sediments, soil and even sand have been isolated and identified (Yasir *et al*., 2009; Poulsen *et al*., 2008; Metcalfe *et al*., 2002a). In this way, each bacterial resident of the same microhabitat may have a distinct ecological advantage (Howard *et al*., 2003a). In bacteria such as *Serratia marcescens* (Suzuki *et al*., 1999), *Aeromonas* sp. 10S-24 (Ueda *et al*., 1995), *Alteromonas* sp. O-7 (Orikoshi *et al*., 2005), *Pseudomonas aeruginosa* K-187 (Wang *et al*., 1998), *Bacillus circulans* WL-12 (Alam *et al*., 1996; Mitsutomi *et al*., 1998), *Streptomyces* sp. J 13-3 (Okazaki *et al*., 1995), and *Streptomyces griseus* HUT 6037 (Itoh *et al*., 2002), the presence of multiple chitinolytic enzymes has been reported. The occurrence of multiple chitinases in a single organism may result in a more efficient use of chitin and its derivatives as a substrate. Moreover, these enzymes can interact in a synergistic way or can complement each other in the light of their different affinities for the substrate, or different regions thereof (Svitil *et al*., 1997). Within the group of bacteria that possess chitinases, an important role is played by members of the *Actinobacteria*, in particular the *Actinomycetes*. These are high-G+C% Gram-positive

bacteria that are often involved in the degradation of different organic materials in natural settings, including chitin and cellulose. Indeed, *Actinomycetes* have been considered to be among the major chitin degraders in soil (Gooday, 1990a; de Boer *et al*., 1999; Metcalfe *et al*., 2002a, Manucharova *et al*., 2007). In this sense, many studies have been performed to pinpoint the chitinase genes from these organisms. Many *Streptomyces* strains possess so-called group A and B family-18 chitinases, and even family-19 chitinases (Williamson *et al*., 2000; Metcalfe *et al*., 2002a). The result of the activity of most bacterial chitin-degradative systems is a collection of short-chain chito oligosaccharides (degree of polymerization $\leq$ 8) including chitobiose. These molecules can diffuse freely into the periplasm of bacterial cells. Interestingly, specific porins for these compounds have been identified in marine *Vibrionaceae* (Keyhani *et al*., 2000).

Considering the chitinase protein families, most bacterial chitinases belong to glycoside hydrolase family 18 (Hoell *et al*., 2010). Within this family, they have been organized in four subfamilies: I, II, III and IV. Based on phylogenetic analyses of the catalytic domains, group I has been further subdivided into groups A, B and C (Watanabe *et al*., 1993). Chitinases of subfamilies II, III, and IV produce (GlcNAc)2 and GlcNAc from chitin, whereas those from subfamily I predominantly produce (GlcNAc)2. Chitinases of subfamilies II, III, and IV also catalyze a transglycosylation reaction, which converts (GlcNAc)4 to (GlcNAc)6 (Takayanagi *et al*., 1991). As indicated in the foregoing, the different activities are often combined in one organism. One unique example is given by *Bacillus licheniformis* strain X-74, which possesses four chitinases, one from each group (I, II, III, and IV) (Takayanagi *et al*., 1991).

Enzyme structure and genetics of bacterial chitinases

Biochemical analyses have revealed that the catalytic domain of bacterial family-18 chitinases always includes a so-called "retention region", which contains one glutamic acid residue, thus functioning on the basis of a retaining mechanism. For several chitinases, in particular from the genera *Streptomyces*, *Shewanella* and *Salmonella*, catalytic domains similar to those existing in family-19 chitinases were found, with two glutamic acid residues and with an inverting mechanism of catalysis. Both family-18 and -19 chitinases require water as a component of the degradation reaction (Karlsson & Stenlid, 2009). The chitin-binding domain can be located either in the amino-terminal or carboxy-terminal domain of the enzyme. This is not unique, as chitin-binding domains have also been found in different proteases and non-catalytic chitin-binding proteins. These domains seem to have the capacity to prevent diffusion of the enzyme or protein away from its intended substrate (Henrissat, 1999). They can bind to the chitin irreversibly, which represents a value for biotechnological applications. For instance, a chitin-binding domain from a *Bacillus circulans* chitinase has been used by New England Biolabs in an expression vector. Besides the catalytic domain, each putative bacterial chitin depolymerase presents an apparent type-II N-terminal secretion signal in addition to accessory domains of

unknown functions like “fibronectin type III” (FN3) and “polycystic kidney disease” (PKD). The domains of complex polysaccharide degrading enzymes are linked by repetitive sequences of polyglycine, serine or threonine, which confer a presumed flexible structure.

The genetic basis of the regulation and synthesis of chitinolytic enzymes has not been completely elucidated yet (Larsen *et al*., 2010; Nazari *et al*., 2011). Several studies indicate that, in general, more than one gene is involved in the regulation of the complex process, which has as a final result a functional chitinolytic system. In spite of the fact that many chitinase genes have been cloned from bacteria from different environments and their biochemical properties clarified (Felse & Panda, 1999a; Henrissat & Davies, 2000), the structure of the presumed operons is still unclear (Delpin & Goodman, 2009). A limiting aspect is also the fact that a majority of naturally occurring bacteria are not cultivable under common laboratory conditions, which severely hampers their study. Moreover, chitin degradation is also influenced by the presence of chitin-binding proteins which do not possess a catalytic domain but act by disrupting the crystalline chitin polymer structure (Vaaje-Kolstad *et al*., 2005).

The low level of certain conserved domains found within bacterial chitinases only allows for the identification of cognate genes for chitin-processing enzymes from environmental samples by using molecular methods. Probes and degenerate primers have thus been constructed based on conserved blocks of amino acids which were identified within the catalytic domains of bacterial chitinases. Using special software like “BlokMaker”, “CLUSTAL-W” (multiple sequence alignment tools) and “Codehop” (primer design), some authors (Hobel *et al*., 2005; Williamson *et al*., 2000) successfully developed PCR-based systems for identification and sequencing of different chitinase genes from nature. The systems have also been used for assessing the diversity of these genes within different bacteria.

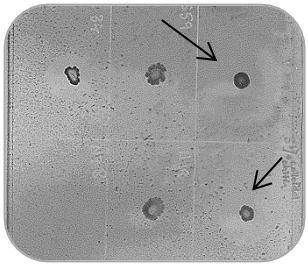
Unfortunately, for the majority of strains that produce chitinases, the organization and regulation of the respective operons is still unknown (Stefanidi & Vorgias, 2008; Suzuki *et al*., 1999; Terahara *et al*., 2009). Whereas we do know that *chiA* and *chiB* genes are main enzymes taking part of the chitinolytic process, these are not the only proteins involved. Indeed, Northern blot analyses have indicated that the *chiA* and *chiB* genes are often part of truly polycistronic operons, yielding strong evidence for the contention that more genes/functions are involved in the process. Primer extension analyses confirmed that transcription of such an operon may start at *chiA* (Morimoto *et al*., 1999). On top of the complexity of the system (operon), there is also a complex regulatory system, which we do not quite understand yet. In the majority of strains analyzed, the presence of glucose was a repressing factor and the system required the presence of chitobiose for induction of the enzymes (Delpin & Goodman, 2009). In fact, one of the first chitinase regulatory systems described in bacteria was the one found in the Gram-positive bacterium *Streptomyces thermoviolaceus* OPC-520. In this system, two proteins that control the level of chitinolytic

activity - ChiS and ChiR – have been found (Tsujibo *et al*., 1999). Among the Gram-negative bacteria, there are only a few studies on the regulation of chitinase gene expression (Chernin *et al*., 1998; Vaaje-Kolstad *et al*., 2005). Recently, it has been shown that also in such bacteria a second chitinase regulatory system is present (Hoel *et al*., 2010; Li & Roseman, 2004; Vaaje-Kolstad *et al*., 2013). A non-catalytic chitin-binding protein produced by *Vibrio furnissii* apparently interacts with a cytoplasmic membrane-anchored regulator (in the absence of the sugar ligand molecule) to repress chitinase gene expression (Li & Roseman, 2004). The presence and binding of chito-oligosaccharides to the chitin binding protein induce a conformational change in the repressor, resulting in signal transduction via phosphorylation of a cytoplasmic response regulator. This then leads to induction of chitinase gene followed by protein expression.

Bacterial chitinases – from chitin-agar plates to screening of metagenomes

Traditionally, biotechnological applications are based on isolation and maintenance in pure culture of targeted microorganisms from environmental samples. Isolation of chitinolytic bacteria from different habitats thus offers an important approach for selecting bacteria with, for instance, high chitinolytic activity under diverse conditions. The highly stable crystalline structure of chitin does not allow its direct uptake by cells. In order to gain access to GlcNAc-oligomers, bacterial cells secrete chitinases (mainly exochitinases) that reduce the degree of polymerization of the substrate. In addition, the presence of small chitin oligomers can induce the bacterial chitinolytic system (Tsujibo *et al*., 1999). Several media have been proposed for the isolation of chitinolytic bacteria from soil and aquatic environments (Hsu & Lockwood, 1975; Souza *et al*., 2009). The success of isolation apparently increases when minimal agar supplemented with colloidal chitin is used (Souza *et al*., 2009; Singh *et al*., 2005). In some cases (e.g. marine samples), such media were supplemented with different salts (Souza *et al*., 2009). Chitin analogs have been well documented for the identification of chitinolytic enzyme-producing strains (Lonhienne *et al*., 2001; Svitil *et al*., 1997). In the assays, chitinase production is observed by visual inspection of cleared zones (haloes) that form around colonies. The main limiting factors of this type of isolation are the quality of the colloidal chitin and the waiting time until haloes are visible. In some cases, such as the isolation of chitinolytic bacteria from arctic samples, long-term incubation at low temperature and high concentration of chitin (up to 5%) was required (Nissinen, Cretoiu & van Elsas, oral communication) (Figure 4).

Figure 4. Chitinolytic bacteria isolated from arctic plant (*Oxyria digyna)* rhizosphere on minimal agar supplemented with 5% chitin, two months incubation at 4^{o}C.



The chitinolytic activity of bacterial isolates can also be evaluated by measuring the production of reducing chitooligosaccharides using chitin-derived soluble substrates (e.g. glycol-chitin, carboxymethyl chitin). Reducing sugars react with a color reagent and absorbance can then be measured. Commercial kits allowing such an analysis have also been developed. Among the most used substrates are 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (a substrate for β-N-acetylglucosaminidase), 4-methylumbelliferyl N,N'-diacetyl-β-D-chitobioside (a substrate for chitobiosidase), 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotriose (a substrate for endochitinase). Although these fluorometric techniques show high sensitivity, the assays remain expensive, time-consuming and most suitable for enzyme extracts than for environmental samples.

Metagenomics and culture-independent developments have opened up a new and dynamic arena in the quest for novel bacterial chitinases. Metagenomic DNA obtained from environments enriched with chitin can be used for the construction of large-insert clone libraries that can be further screened using function-based or sequence-based strategies. Direct sequencing of metagenomic DNA isolated from different environments has shown the potential of this methodology in retrieving novel glycosyl hydrolases (Li *et al*., 2009). A key issue in metagenomic screening is the selection of the proper environment. Although quite a number of characterized bacterial chitinases reported in the CAZy database are produced by aquatic organisms, soil is regarded to constitute the most important environment for mining of novel chitinases (Berlemont & Galeni, 2010; Hjort *et al*., 2010; Cretoiu *et al*., 2012). Soils harbor a highly diverse microbial community and constitute major reservoirs of active enzymes and their genetic background. The bacterial diversity in soils in many cases exceeds that of other environments. Thus, a great number of novel enzymes has already been obtained from soil using metagenomics (Daniel, 2004; Jeon *et al*., 2009; Nacke *et al*., 2011; Nacke *et al*., 2012). A recent study (Nacke *et al*., 2012) reported the identification of one cellulase and two xylanase genes derived from a soil metagenome. Expression and characterization of these novel enzymes revealed interesting properties, such as a high activity in a broad range of temperatures and pH values and halotolerance. Furthermore, comparative metagenomic and physiological analysis of soil microbial communities across nitrogen gradients (Fierer *et al*., 2012) included genes for chitin and chitin-oligomer degradation in the analyses. Direct sequencing (Illumina HiSeq)

of pooled fosmid clones bearing large inserts of metagenomic DNA isolated from a chitin-amended soil indicated a large proportion of putative glycosyl hydrolases (Figure 5, Cretoiu *et al*., in preparation).

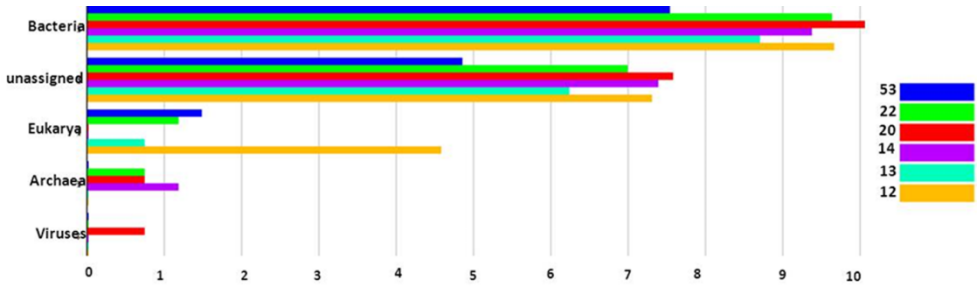


Figure 5. Domain distribution of sequences (%) affiliated to putative chitinases. Sequences (Illumina) obtained from pooled fosmid clones with 40 kb inserts of metagenomic DNA from chitin-amended soil. Annotation based on comparison with sequences of GH18 and GH19 retrieved from CAZy. Sample (pool) name depicted by color.

Considering the successes (hit rates) of metagenomics approaches to soil microbial systems, these have often been low (Daniel, 2004; Uchiyama & Miazaky, 2009). Hence, whereas libraries can be readily constructed from soil DNA, the required library screenings can be tedious and hit rates can be disappointingly low. Hence, the concept of "***Ecological enhancement***", which implies the in-situ positive biasing of the system, for instance by incubation with the desired substrate and under selected conditions, was recently brought forward as an aid to metagenomics approaches (Ekkers *et al.*, 2012). Such a deliberate manipulation of microbial communities via ecological enhancement by chitin amendment offers possibilities to shift the balance in the functional chitin-active genes and enhance the chance of recovering such enzymes.

Aim of this thesis and research questions

The aim of this study was to gain access to the chitinolytic potential borne in the cryptic bacteriota of natural habitats, in particular soil, by making use of metagenomics tools. It was felt that such an approach would not only enhance our understanding of the successional chitinolytic processes in such systems, but also enhance the possibilities for mining of novel chitinolytic enzymes with enhanced features that can be further bioengineered.

Most studies conducted so far indicate that the diversity of bacterial chitinolytic enzymes is the consequence of different environmental cues. Although the degradation of chitin is an important first step in global nutrient cycles, production of chitinases is probably not essential for heterotrophic bacteria living in most environments. This is because many other carbon and nitrogen sources are often available. On the other hand, it is logical to assume that under chitin selection, particular (heterotrophic) bacteria will activate their chitinolytic systems and thrive under the conditions applied. Such conditions may be set by the experimenter, but are also locally determined, by the microbiota itself. It is a challenge to obtain a deeper understanding of what is going on locally in the chitinoclastic process and at the same time have an open eye for potential exploration of the systems involved. One such application lies in the area of *in situ* disease suppression, and in finding a link between the effects of chitin added to soil, the microbial community shifts and the chitinoclastic organisms that come up in such a biased environment (Beier & Bertilsson, 2013; Hjort *et al*. 2006; Metcalfe *et al*., 2002b;).

In general, there is a perceived need to improve the understanding of the natural prevalence of the organisms and enzymes involved in the chitin-degradative process and how these are driven by environmental cues, in the light of future agricultural as well as biotechnological applications.

With reference to the aforementioned arguments, the general hypotheses of this study are:

- Different types of habitats drive bacterial communities to the synthesis of different chitinolytic systems.
- The ecology and evolution of bacterial chitinolytic enzymes is related not only with the presence of chitin, but also with the conditions of the habitat in which these reside.
- Changes within bacterial communities incited by chitin can modulate the intrinsic capacities of soil systems, such as the suppressiveness towards plant pathogens.
- Functional metagenomics screening of chitin-amended soils represents an important step on the route to finding novel chitinases.

With respect to these, the following general research questions were derived:

- How different are natural habitats in terms of the complement of bacterial chitinases? What are the relationships between the diversity of the bacterial communities and the chitinolytic activity?
- To what extent do bacterial communities change in soil with added chitin? How does this relate with alteration of other local environmental conditions, such as pH?
- What are the chitin-responsive soil community members? How does the response relate to practical applications like induction of suppressiveness towards plant pathogens?
- What are the molecular features of chitinolytic systems from bacteria that prevail under added chitin? Does the use of functional metagenomics screening assist us in mining for novel chitinases?

Outline of the thesis

The thesis is divided in the following sections, in which specific hypotheses and research questions are treated in more detail, chapters 2 to 9.

Chapter 2 gives an overview of function-based metagenomics assessment strategies for exploration of microbial communities for active enzymes and biomolecules. Metagenomics experimental methodology is described and limitations are discussed. Practical issues such as sample selection, DNA extraction, metagenomic library construction and gene expression are discussed.

Chapter 3 addresses the metagenomic exploration of disease-suppressive soils and provides a comparison of methods that can be used. The putative relationship between soil chitinolytic activity and soil disease suppressiveness is discussed.

Chapter 4 provides an experimental evaluation of chitin-degrading microbial communities from well-known and still underexplored terrestrial and aquatic habitats. A combined protocol, which encompasses classical enzymatic assays and *chiA* genetic screening, is applied. The first deep *chiA* based pyrosequencing study is reported. The promise of several habitats for further bioexploration is discussed and selected ones are brought forward.

Chapter 5 examines the changes in a bacterial chitinolytic community in a short-term soil microcosm experiment in which chitin and pH levels were modified. A major role of Gram-negative bacteria such as *Oxalobacteraceae* versus a smaller one of *Actinobacteria* in the immediate response to the added chitin source was indicated.

Chapter 6 investigates the actinobacterial and oxalobacteraceal communities under chitin amendment in an agricultural soil. For this, a key agricultural soil from a field established in the Netherlands in 1955 was selected. Significant changes in the *Oxalobacteraceae* as a result of chitin addition were observed and new insights into the effect of chitin addition on soil disease suppressiveness provided.

Chapter 7 presents a 16S rRNA gene based pyosequencing analysis of the chitin-amended

field soil. The response of some bacterial groups, in particular *Actinobacteria* and *Oxalobacteraceae*, was consistent with previous findings. The data support the use of the chitin-amended field soil for further screening for bacterial chitinases.

Chapter 8 describes the screening of a large metagenomic fosmid library constructed on the basis of total DNA isolated from the chitin-amended field soil. Full-length insert sequencing of selected fosmids confirmed the existence of putative novel bacterial chitinase genes. *De novo* annotation and general characteristics of the genetic fragments recovered are presented. The analysis provided a view of chitinase genes useful for further expression and biotechnological applications.

Chapter 9 discusses the overall results obtained in the experimental and theoretical approaches, with a focus on the significance of the methods applied and data gained. Further research prospects related to chitin, bacterial chitinases and the use of metagenomics are provided.